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Comparative investigations concerning the significance of the mode of infection in experimental anthrax.

by G. Sobernheim and H. Murata.

Zeitschrift f. Hygiene, 103: 691-698 (1924).

In a recently published paper, Tada proved that the immunization of guinea pigs against anthrax is largely unaffected by the type of inoculation, provided the same vaccine is utilized in identical doses. In particular, the results obtained in connection with cutaneous instillation of the vaccines did not differ from those produced by subcutaneous or intramuscular injection, not even when a direct infection of the skin was positively avoided in the latter case. These tests therefore proved that immunity to anthrax is not dependent upon the epidermis, but may be conferred in the same manner by way of other tissues. This disclosure agrees with the general experience and with earlier experiments, but does not correspond to concepts expressed in recent times, particularly by Besredka.

Although the results of aforesaid experiments already pointed to identical circumstances in the case of anthrax infection, it nevertheless seemed indicated to illuminate the question once more through special tests.

It has been known for some time that the portal of entry of the anthrax pathogen, be it in the form of bacilli or spores, is not irrelevent to the course of infection, and even R. Koch and his co-workers have shown in their earliest research that a successful stomachal infection, for instance, in which the anthrax spores penetrate through the intestinal mucous membrane, requires considerably greater quantities of bacteria than would be needed for subcutaneous infection. Later diverse other authors (Noetzel, van Leent, and others) have stressed the difference in effect displayed by subcutaneous, intraperitoneal or intravenous injection of bacteria, and there can be no doubt about the fact that the peculiarities of the tissue in which the intruding or injected anthrax bacilli first attempt to gain a foothold are, to a certain extent, important. This circumstance will be clearly understood if we take into consideration the fact that the fate of the causative agent depends primarily on the extent to which the natural defensive substances of the organism, humoral and cellular, are able to become effective from the start. Thus the conditions are distinctly more favorable to the organism in the case of intravenous and intraperitoneal infection than in infections emanating from the subcutaneous connective tissue, where the bacteria are not as accessible to the influence of defensive substances. Similarly, cutaneous inoculation initially shields the bacteria somewhat from the defensive reaction of the infected body. It follows that successful infection requires different quantities of bacteria, depending on the mode of infection (provided the virulence is identical), and it is therefore indispensable that the strictest attention be paid to the dosage in all tests which attempt to determine the susceptibility of various tissues. It is impossible to clarify the present problem without considering the quantitative basis. When we observe that a dose of virulent anthrax culture, which

positively kills guinea pigs after cutaneous or subcutaneous infection, is ineffective when injected intraperitoneally or intravenously, this does not indicate that the latter modes of infection are unsuitable, but that the dose is to be increased correspondingly.

The concept of dosage has not always been sufficiently considered in recent tests. Besredka, in particular, who sees the epidermis as the body's sole vulnerable point in anthrax infections and considers anthrax only as a dermal infection, bases his opinion on tests which, in this respect, are lacking far-reaching and, as we shall see, decisive variations in the infective doses. Anthrax is said to develop only upon cutaneous infection, at least in guinea pigs and rabbits, and if other modes of infection cause fatal anthrax, this circumstance is always claimed to be connected with simultaneous injury and infection of the skin. Besredka has formulated his concept of the mode of anthrax infection to the effect that an animal would possess total immunity to anthrax if it were possible to skin it and to keep it alive, since the anthrax pathogen could no longer be caused to take hold (see also Delater).

Besredka's conclusions have provoked concurrence (Aitoff, Balteano, Brocq-Rousseau and Urbain), but also contradiction or limitation (Combiesco, Boquet, and others). Bachmann, Beltrami and Romat have been able to cause anthrax in rabbits by subcutaneous and intravenous injection, while carefully avoiding cutaneous infection, when the culture dose was slightly raised; they disclaim the favored disposition of the skin championed by Besredka, at least as far as the anthrax strains utilized by them are concerned. Gratia also achieved positive results in connection with intravenous infection, but different types of anthrax bacteria seem to exhibit irregular behavior. Whether simple differences in virulence or biological variations are involved, cannot be deduced with certainty from his tests.

We proceeded in our own investigations by infecting a large number of guinea pigs with precisely measured quantities of bacteria intracutaneously, subcutaneously, intramuscularly, intraperitoneally and intravenously. The starting material consisted of invariably recent agar cultures of a virulent anthrax strain, about 20 hours old. These were used in the production of suspensions in physiological saline initially in dilutions of 1 loop per 10 ccm. This suspension was passed through a sterile paper filter to remove larger particles, and then diluted further. The animals always received 0.1 ccm of the pertinent dilution.

Inoculation was accomplished by intracutaneous injection into a previously depilated spot of the abdominal skin. All other types of infection were according to a method which excluded any dermal infection. First a small cut was made in the skin, the wound was held open with blunt hooks, then the injection was made with a most careful and positive avoidance of the skin. Afterwards the point of puncture and the edge of the wound were encrusted with a glowing glass rod and brushed with collodium. The fact that a direct penetration of anthrax germs into the skin is thus completely prevented is shown by the absence of edematous infiltrations at the site of inoculation, especially in the case of intravenously (v. jugularis) and intraperitoneally infected animals. Without exception the various infective attempts were

undertaken simultaneously on several animals (4-6), in order to prevent possible coincidences.

The result of inoculations thus carried out with calibrated amounts of virus is evident from the following table. The table shows that every type of infection was capable of causing fatal anthrax. The animals' death as a rule occurred after 2-3 days; the post-mortem findings were typical. It is inconceivable that the skin represents the sole vulnerable tissue of the guinea pig's organism, since the remaining modes of infection are also effective. It was again confirmed, as was to be expected and as had been known from previous experimental tests, that different amounts of bacteria may be required for lethal infection, depending on the manner of application. According to our results, there is no difference between intracutaneous and subcutaneous inoculation, at least not in favor of the cutaneous mode. For the two guinea pigs infected subcutaneously promptly succumbed to infection with 1/100,000 loop, and only 1/1,000,000 loop had an irregular effect, while intracutaneous inoculation with 1/100,000 loop already proved uncertain. In the successful intramuscular infection, the dose of 1/1,000,000 loop was positively lethal in the same series, making this mode of infection even superior to cutaneous infection, intracutaneous or subcutaneous. In the case of intraperitoneal injection of bacteria, the positively lethal minimum dose was considerably higher, in that only one of two animals died from 1/10,000 loop. The same amount of bacteria was positive in connection with intravenous inoculation.

Table.

Determination of the minimal lethal dose of virulent anthrax culture for guinea pigs with different modes of infection.

| No. | Weight in g | Mode of infection | Dosis in loops | Result |
|---|---|---|---|---|
| 1 | 390 | intracutaneous | 1/100,000 | ✗ |
| 2 | 500 | " | " | lives |
| 3 | 350 | " | 1/1,000,000 | ✗ |
| 4 | 600 | " | " | lives |
| 5 | 420 | subcutaneous | 1/100,000 | ✗ |
| 6 | 500 | " | " | ✗ |
| 7 | 450 | " | 1/1,000,000 | ✗ |
| 8 | 550 | " | " | lives |
| 9 | 420 | intramuscular | 1/100,000 | ✗ |
| 10 | 500 | " | " | ✗ |
| 11 | 480 | " | 1/1,000,000 | ✗ |
| 12 | 630 | " | " | ✗ |
| 13 | 530 | intraperitoneally | 1/10,000 | ✗ |
| 14 | 530 | " | " | lives |
| 15 | 490 | " | 1/50,000 | ✗ |
| 16 | 530 | " | " | lives |
| 17 | 420 | " | 1/100,000 | lives |
| 18 | 420 | " | " | lives |
| 19 | 380 | intravenous | 1/10,000 | ✗ |
| 20 | 380 | " | " | ✗ |
| 21 | 400 | " | 1/50,000 | lives |
| 22 | 400 | " | " | lives |
| 23 | 390 | " | 1/100,000 | lives |
| 24 | 400 | " | " | lives |

Intramuscular infection was the mode which succeeded easiest, i.e. with the smallest bacterial dose. Subcutaneous inoculation was nearly as favorable, less so the intracutaneous method, and only the intravenous and intraperitoneal injections required a considerably more potent infection.

Mention should be made also of a few tests with mice (subcutaneous and intravenous infection) that followed our experiments with guinea pigs. They showed the same essential results. In the case of intravenous inoculation, we utilized the old system chosen by Schimmelbusch of inoculating with a thin canula at the very tip of the tail and amputating the tail far above the point of puncture immediately thereafter. The injection invariably amounted to 0.1 ccm. After amputation the wound was thoroughly encrusted. Subcutaneous inoculation was undertaken on the abdominal skin, as on the guinea pig, with careful prevention of any direct cutaneous infection. The result revealed that the subcutaneously inoculated animals without exception succumbed to anthrax in 2-3 days up to doses of 1/1,000,000 loop, and that even 1/5,000,000 loop proved fatal, even if with less certainty. On the other hand, the minimal lethal dose for intravenous injection amounted to 1/100,000 loop. It is therefore easy to induce anthrax in mice without cutaneous infection, and in these animals, as in the guinea pig, larger amounts of bacteria are required in the blood stream than in the subcutaneous connective tissue.

The results of the discussed tests are clear and unequivocal. We had originally considered still another test method and had tried it in a few experiments. Precisely measured amounts of our bacterial suspension were fused into thin glass capillaries and implanted under the animals' skin. The capillary tube was introduced into the subcutaneous connective tissue through a cut in the abdominal side of the guinea pig and pushed under the skin as far as possible. The wound was then closed and after healing (about 5 days later) the glass capillary which had been absorbed without reaction, was broken from the outside. All animals became infected with anthrax and had edematous infiltrations of the skin at the site of the capillaries; the animals succumbed to anthrax 2-3 days later. We decided not to continue and evaluate such tests because an injury and direct infection of the covering skin may easily occur during the breaking of the glass capillary, and the tests seemed objectionable in the sense of the present argument.

However, another test series, carried out as a supplement to the one just discussed, clearly contradicts the concept of the exclusive susceptibility of the epidermis. These are tests with stomachal infection of the animals. Intravenous, intraperitoneal, intramuscular or subcutaneous injections, as carried out by us, always presuppose a direct injury to the skin, and even if we had completely eliminated the possibility of a direct dermal infection, one could still argue that the injected anthrax bacteria, now spreading through the lymphatic and hemal channels, may indirectly reach the injured spot in the skin and thus cause the cutaneous infection proper, as postulated by Besredka and his supporters. Indeed, this objection has recently been raised in a paper by Plotz, published after the conclusion of our tests, in which he reports on results which, similarly to ours, speak for equality among the different infective modes, but where he nevertheless ascribes the decisive importance to the epidermis. He bases this on the

points of view just expressed and claims to have proved it by the capillary method which is said to infect the test animal (rabbit) successfully only as long as the skin is not completely scarred. When he broke the bacterial capillaries after 6-7 days, infection no longer succeeded regularly. The animals dying in this case in his opinion succumb to anthrax due to a cutaneous infection caused by the breakage. Aside from the fact that the latter explanation begs the question, the whole infective mode is technically objectionable, as admitted by Plotz himself, and therefore unsuitable for the gaining of unequivocal results. His results permit another explanation: The capillaries were filled with broth cultures, and it is certain that these cultures did not possess the same virulence after 6-7 days as young, 24-hour cultures. The controls to this effect imposed by the author are not necessarily valid due to insufficient precision in dosage.

For infections per os we used older agar cultures (2-3 days) which had sporulated fairly well, and infected a series of guinea pigs so that measured amounts were introduced into the animals' stomach with a pharyngeal probe. These tests also gave expected results and confirmed the old experience that anthrax may be caused in susceptible animals by feeding them spores. The lethal dose was considerably higher than in parenteral infection, but 1/10 loop caused a positively fatal infection. The animals died on the 4th day with the characteristic lesions of the intestinal mucous membrane. Numerous points on the mucous membrane revealed small, pinhead to pea-sized furuncular ulcers representing the portals of entry and the primary sites of bacterial attack.

Our experiments in their totality reveal the fact that it is possible to infect guinea pigs with a virulent anthrax culture, not only cutaneously but also subcutaneously, intramuscularly, intravenously, intraperitoneally and stomachally, and that a primary infection of the skin is not necessary for the genesis of anthrax. Our tests are in full agreement with the results obtained by Tada in his immunological experiments. Similarly, we should like to mention pulmonary anthrax in this connection. The fact that an anthrax infection may develop in the lungs, without primary involvement of the epidermis, is attested by animal tests and clinical observations of man. It represents an additional example which teaches that diverse organs of the body are vulnerable to an anthrax infection, and that this susceptibility is by no means restricted to the skin.

The dosis letalis minima of a virulent anthrax culture is subject to rather extensive fluctuations, depending on the mode of application. This is easily understood. For instance, obvious difficulties, so well known to us, are encountered by the bacteria during penetration of the intestinal mucous membrane, applicable even to those pathogenic types which display a typical affinity to the intestinal mucous membrane (typhus, dysentery, cholera). The fact that intraperitoneal or intravenous infection requires greater amounts of bacteria than cutaneous or subcutaneous inoculation, is easily explained (as previously mentioned) by the circumstance that here the bacteria meet the whole influence of the organism's defensive forces, and that the phagocytic effect, especially, is able to develop its full power. All this occurs more slowly and less completely in the skin, enabling the

pathogen to adhere more easily. The correctness of this concept may be demonstrated by an artificial elevation of the skin's resistance brought about by strong leukocytosis. First we inoculated the guinea pigs subcutaneously with a sterile suspension of aleuronate in broth (2.5 ccm), then, 18 hours later, with an ordinarily lethal dose of our anthrax culture (1/100,000 loop) at the same site, again subcutaneously. It was noted that the animals now survived the infection, while the control animals infected simultaneously without pre-treatment died, as usually, after 2-3 days (Gratia has conducted similar experiments recently). This result is hardly surprising. It thoroughly agrees with the knowledge gained since R.Pfeiffer's basic cholera investigations concerning non-specific increase in resistance. At the same time, these observations contain a renewed indication of the necessity to differentiate such manifestations from genuine immunity. Recent reports on successful local tissue immunization, leading to the development of immunity, without formation of antibody, within 24 hours, require more thorough analysis before they are accepted as proof of a true tissual immunity.

These conditions, applicable for the time being only to anthrax infections, are, in our opinion, of principal significance. For anthrax ought to represent the classic example of strict localization of infection and immunity. The proof that the epidermis does not play this decisive role in anthrax infections, should therefore be of general interest. Every observation that offers clarification in one direction or another is important for the question concerning the general or local character of infection and immunity, studied nowadays in connection with many infectious diseases from new points of view. As far as anthrax is concerned, susceptibility or immunity of an individual is not simply linked to the properties of a certain single organ.